Final report for PD105027 OTKA project

The aim of this project was to establish a measurement which can measure the fidelity of RNA-dependent RNA-polymerase (RdRp), mutagenize our strain of type 1 PRRSV (HU14432/2011), and using the fidelity measurement method, establish a strain of which mutagenesis ratio is 10-100 times lower than that of the wild type. The project – originnaly planned for three years – was terminated after 1 year 2 months of work because the PI got a chance to participate in an international EU-funded project, and there was no possibility to hand over the grant to third party.

Proposed aims

For this project we proposed the following aims.

- We would determine type 1 PRRSV RdRp fidelity and to develop an at least 10-100 fold higher fidelity RdRp PRRSV strain that might be used to stabilize PRRSV MLV constructs.

- We would create a selection system based on recombinant type 1 PRRSVs that contain point mutant variants of enhanced green fluorescent protein (eGFP). The mutants – termed eGFP0 – have no green light emission capability.

- We would do mutagenesis to mutate the RdRp of eGFP0 PRRSVs and measure the reversion (eGFP0→eGFP) rate of these viruses by detecting green light emission in the back mutant PRRSV-infected cells, so we would be able to select RdRp mutants with 10-100 fold higher fidelity.

Results

Cloning type 1 PRRSV

As the first step of cloning we determined the complete sequence of the strain HU14432/2011 along with an other – newly described – Hungarian isolate 9625|2012. A paper of the sequences of the two strains along with bioinformatical analysis (title: Full-length genome analysis of a Hungarian porcine reproductive and respiratory syndrome virus isolated from severe respiratory disease.) is submitted for publication, but not accepted yet. We made a 6 step assembly strategy for cloning, but only the first step of the assembly was done before project termination.

Creating eGFP and eGFP0 type 1 PRRSV strains

In parallel to cloning of type 1 PRRSV, we used the EGFP-PRRSV of North American origin clone pSD01-08-GFP (Fang et al 2006), that was possessed by our lab, to set up methods. We started to create eGFP0 strains based on this strain using the long overlapping PCR strategy.

In the first few months of the research it became clear that the PCR-based method developed by our lab is more template dependent than it was expected. Despite our efforts to optimize it, most of the viral DNA samples from different sources failed to amplify. Since our approach required several similar cloning steps (to establish a series of clones), we had much effort on increasing the success rate. However at one point it became clear that we need to use standard techniques to build our strains.

To measure fluorescent cell number we acquired the essential equipment of the project, a Millipore Muse cell analyzer (a simple flow cytometer) in January 2013. Our plan was to do this purchase by November 2012, but we were obliged to do the purchase via tender because the institute had already reached a limit of money that could be spent without it.
In our original plans it was a possibility to buy a second hand device (there are resellers and a complete market based on used scientific equipments), but the tender excluded this option. Only the Millipore device remained that did fit the budget, but it was incapable to measure eGFP, so we made a compromise to buy this cytometer and use one of the red versions of GFP (dsRed) instead of eGFP. This meant however that our eGFP based system must have been redesigned into dsRed and no eGFP clones can be measured. We obtained a dsRed containing plasmid (pDSRED-monomer-N1) and made two point mutants of the dsRed gene. Our attempts to change eGFP into dsRed in the clone pSD01-08-GFP was not successful until project termination. However we used dsRed transfected cells to test the flow cytometer and the immunofluorescent (IF) labeling of this protein.

Creating RdRp mutant eGFP0 PRRSV strains

We proposed 3 different approaches to create these mutants:

1. Random mutagenesis using RNA nucleotide analogues.
2. Structural similarities of known high fidelity RdRp mutants.
3. Predicting and altering so-called chameleon sequences. (These sequences have no strong preference to form neither alpha-helix, nor beta-sheet, our idea was that changing them to a sequence with a stronger preference can affect the overall flexibility of the protein and might increase the fidelity.)

For Approach 1 (random mutagenesis) we used a method described in ref (Beaucourt et al. 2011) that we attempted to implement. The theory of these strategy is based on two observations:

(1) the viral population can not effectively survive if the genome is too unstable and

(2) the mutagenesis ratio caused by the infidelity of the wild type RdRp is close to this limit.

If we increase the mutagenesis rate by mutagen treatment, an thus, reduce genome stability, the virus will respond with an increased fidelity RdRp. In the first step however, the viral population collapse (but still survive), and it takes several passages for the virus to evolve a high fidelity RdRp and reach its original viability. We used 5-azacitidne, 5-fluorouracil and ribavirin as well as the equimolar mixes of them. The protocol assumes that there is a range of mutagen concentrations which is low enough to be acceptable for the host cell but high enough to reduce the viability of the virus by at least 1 log. The mutagenesis can be carried out easily if this assumption is true, so the infected cell culture can be treated with the acceptable concentration of the mutagen to collapse the viral population. The endpoint of the measurement is measured by titration (the virus titer should return to the original state). This is a well established system used on several RNA viruses.

In our system the maximal acceptable concentrations (10 mM of each of the mutagens) were not enough to reduce viral growth. Higher (20 mM) concentrations however led to reduced cell growth (maximum 30-40% of confluence) as well as reduced viral viability.

Reduced host cell viability, per se, can lead to reduced number of infected cells: both the number of fluorescent foci that we wanted to follow in the original plan and the size of them were reduced, the latter is probably because the virus cannot infect via cell-cell junctions.

Therefore we modified the original titration-based following of viral viability and designed a quantitative PCR (qPCR) based method to follow the viral genome copy number and calculate ratio of fluorescent foci and copy number. The theory is that a low titer can be caused by low number of high virulence viral particles, as well as high number of low virulence viral particles. In the first case the ratio of viral genomes and virus foci is high, in the latter case it is low. In this system we could follow the ratio of viral genome and fluorescent viral loci.

Here we have to mention that the in the first times the reproducibility of the infection and titer measurement with our infective strain was unexpectedly low. Since our measurement is based on virus titer measurement, it was essential to implement a method which gives a reproducible titer (deviation within 1 log) of the same virus stock. Our initial attempts resulted in ~5 log differences of measured titer. After changing all of our reagents, tissues, and after technical cooperation with the PRRSV lab of the National Food Chain Safety Office, the issue could not been solved. As for titer measurement we followed the EGFP transgene, our most probable explanation was that EGFP can be unstable in the viral genome and this is why in some cases we cannot see infection while in other cases we see high titer of the same sample. After failing to measure titer by EGFP we established an immunoflurosecence (IF) based method using antibody against capsid proteins which showed the same reproducibility issue.

Finally we came to the conclusion that instead of PRRSV capsid we must label the fluorescent protein. We choose this solution because of two reasons: (1) our experiments to label PRRSV itself with IF assays remained unreliable and (2) by using this solution we can avoid to measure infected cells that do not contain any (mutant or wild type) flurescent protein. As the stability of modified PRRSV clones are always a question, this method allows us to measure the correct ratio of mutant and revertant fluorescent protein and avoid to measure false cells which are infected with PRRSV that had lost the fluorescent gene. At the time of this decision we had already changed our strategy from eGFP to dsRed, therefore we obtained anti-dsRed primary antibody and carried out the first immunofluorescent (IF) experiments to label dsRed-containing cells.

For approach 2 (mutagenesis using structural similarities) we got in contact with bioinfarmitcs experts to offer collaboration. For approach 3 (finding chameleon sequences) we used a chameleon sequence predicting program to find candidate sequences to modify. The prediction resulted in a high number of candidate sequences, and it seemed to be more effective if we integrate these two approaches and use structural similarities to reduce the number of candidate chameleon sequences. For these approaches finally we came to the conclusion that we should postpone them until we see the results of random mutagenesis.

Challenges solved after the first 15 months:

- PRRSV mutagenesis to obtain high RdRp fidelity cannot be done on the way that was published. We modified the method and developed a qPCR based method to follow the ratio of viral genome and viral foci.
- Instead of using the PCR based cloning (which challenge remained unsolved) we started to use conventional cloning of the HU14432/2011 strain. As a first step of it, we determined the complete sequence of the strain and an onther Hungarian strain 9625|2012. The sequence results and their bioinformatical analysis is under publication.
- We rebuilt our strategy of using eGFP into using dsRED. We carried out the first dsRED based experiments containing dsRED mutagenesis, IF and flow analysis.

References

Beaucourt S, Borderia AV, Coffey LL, Gnadig NF, Sanz-Ramos M, Beeharry Y, Vignuzzi M (2011) Isolation of fidelity variants of RNA viruses and characterization of virus mutation frequency. *J Vis Exp*.

Fang Y, Rowland RR, Roof M, Lunney JK, Christopher-Hennings J, Nelson EA (2006) A full-length cDNA infectious clone of North American type 1 porcine reproductive and respiratory syndrome virus: expression of green fluorescent protein in the Nsp2 region. *J Virol* **80**, 11447-11455.